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Treatment for prostate cancer depends on multiple factors including the stage of the tumor and expression of the androgen receptor (AR). Endocrine therapy can be used for treatment of early stage androgen-responsive tumors, whereas chemotherapy for later stage androgen-nonresponsive tumors is problematic. We have investigated the aryl hydrocarbon receptor (AhR) as a potential target for treating prostate cancer using a new series of relatively non-toxic selective AhR modulators (SAhRMs). Initial studies show that 22RV1, PC3 and LNCaP prostate cancer are Ah-responsive and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces CYP1A1-dependent activities in all three cell lines. Moreover, two SAhRMs, namely diindolylmethane (DIM) and 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) inhibit growth of AR-positive 22RV1 and AR-negative PC3 prostate cancer cells. In addition, AhR ligands inhibit dihydrotestosterone-induced upregulation of AR protein in 22RV1 cells suggesting a possible mechanism for inhibitory AhR-AR crosstalk. The growth inhibitory effects of SAhRMs in PC3 cells suggests that AhR ligands also inhibit growth of androgen-nonresponsive cells.

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INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer in North American men and it is estimated that there are over 300,000 newly diagnosed cases each year (1, 2). The incidence and mortality rates from prostate cancer are increasing and this is due, in part, to an increasingly aging population and the higher incidence of this disease in older men (3, 4). Prostate cancer therapy is dependent on the stage of the tumor and androgen receptor (AR) expression. Early stage AR-positive prostate cancer is responsive to endocrine therapy; however, later stage disease requires more aggressive chemotherapeutic regimens. Development of new mechanism-based drugs has been described and these compounds should provide needed improvements for treatment of both AR-positive and AR-negative prostate tumors (5-8).

A recent study in androgen-responsive LNCaP prostate cancer cells showed that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a ligand for the aryl hydrocarbon receptor (AhR), inhibited testosterone-induced cell proliferation and gene/reporter gene expression (9). Research in this laboratory is focused on development of selective AhR modulators (SAhRMs) that exhibit tissue-specific AhR agonist or antagonist activity (10, 11). Alternate substituted (1,3,6,8- or 2,4,6,8-) alkyl polychlorinated dibenzofurans, typified by 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF), are relatively non-toxic and inhibit prototypical AhR-mediated toxic responses in rodent models (i.e. AhR antagonists) but exhibit selective AhR-dependent antiestrogenic and antitumorigenic activities in mammary tumor models (12-16). 6-MCDF also inhibits growth of some ER-negative breast cancer (16) and pancreatic cancer cells (17). Results of studies summarized in last year's report showed that 22RV1 and PC3 prostate cancer cells

were also Ah-responsive and both MCDF and diindolylmethane (DIM) inhibited prostate cancer cell growth. In addition, TCDD modulated dihydrotestosterone-induced AR-expression in 22RV1 cells. This report summarizes recent studies with TCDD and 6-MCDF in LNCaP prostate cancer cells. Both compounds inhibit cell growth and E2- and androgen-induced transactivation in LNCaP cells transfected with an androgen-responsive construct containing probasin gene promoter inserts. Effects of various treatments on AR protein expression were variable.

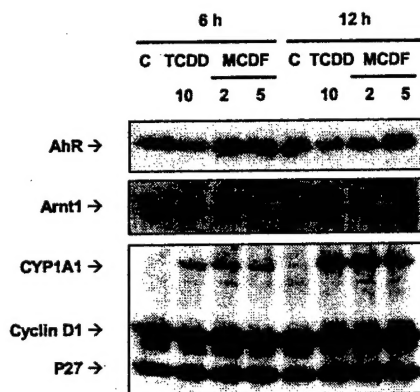
BODY

Effects of TCDD and 6-MCDF on AhR Activation and Growth of LNCaP Cells

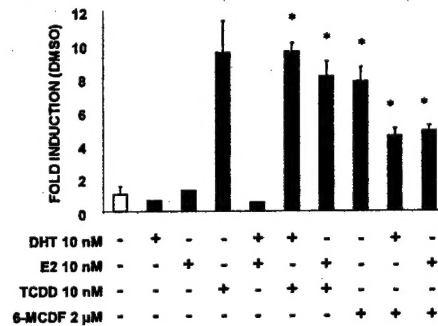
Previous studies reported that the AhR and Arnt mRNA are expressed in LNCaP cells and Ah-responsiveness was confirmed by induction of CYP1A1 mRNA and CYP1A1-dependent EROD activity by TCDD (9). Results illustrated in Figure 1A show that 10 nM TCDD, 2 and 5 μ M 6-MCDF induce CYP1A1 protein in LNCaP cells, and this is consistent with previous reports showing that 10 nM TCDD induces CYP1A1-dependent EROD activity (9). Western blot analysis also confirmed expression of both AhR and Arnt proteins, and treatment with TCDD but not 6-MCDF decreased expression of the AhR. Expression of other proteins including Sp1, cyclin D1 and p27 were unaffected by the treatments and serve as loading controls. Results illustrated in Figure 1B also show that treatment of LNCaP cells with 10 nM TCDD induced luciferase activity > 9-fold compared to solvent control (DMSO) in cells transfected with pDRE₃. In contrast, 10 nM DHT, 10 nM E2 and E2 plus DHT did not significantly induce activity, and neither DHT or E2 in combination with TCDD affected induced activity. 6-MCDF (2

μM), a prototypical SAhRM, also induced luciferase activity (> 7 -fold), and this was consistent with the induction of CYP1A1 by 6-MCDF. 6-MCDF is a much less potent agonist for activation of CYP1A1 or DRE-dependent activities in breast cancer cells (12). Both E2 and DHT in combination with 6-MCDF significantly inhibited 6-MCDF-induced activity, whereas in cells treated with TCDD in combination with E2 or DHT, inhibitory interactions were not observed.

A. LNCaP cells



B. pDRE₃



C.

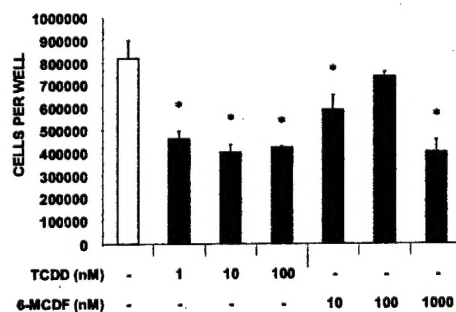


Figure 1. Ligand-dependent AhR activation and growth inhibition in LNCaP cells. [A] Induction of CYP1A1 protein. LNCaP cells were treated with DMSO (C), 10 nM TCDD, 2 or 5 μM 6-MCDF for 6 or 12 h, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. Antibodies were used to detect the AhR, Arnt, CYP1A1, cyclin D1 and p27 proteins. [B] Activation of pDRE₃. LNCaP cells were transfected with pDRE₃, treated with various compounds and luciferase activity was determined as described in the Materials and Methods. Significant induction ($p < 0.05$) is indicated with an asterisk and inhibition of TCDD- or 6-MCDF-induced activity is also indicated (**). [C] Inhibition of LNCaP cell growth by TCDD and 6-MCDF. Cells were cultured for six days, treated with different concentrations of TCDD or 6-MCDF, and cell numbers were determined as described in the Materials and Methods. Significant ($p < 0.05$) growth inhibition is indicated by an asterisk. All results are presented as means \pm SE for three replicate determinations for each treatment group. Growth inhibition in some of the groups was observed after 2 to 4 days.

The comparative effects of TCDD and 6-MCDF on growth of LNCaP cells were also determined in cells treated with solvent control and different concentrations of the

AhR agonists for 6 days. The results show that TCDD (1 – 100 nM) significantly inhibited proliferation of LNCaP cells, and growth inhibition was also observed for 6-MCDF (Fig. 1B). Both compounds inhibited $\geq 50\%$ cell growth at one or more concentrations. Similar experiments were also carried out with 6-MCDF and TCDD in LNCaP cells also treated with different concentrations of DHT (up to 10 nM). Hormone-induced cell growth was not observed; however, both 6-MCDF and TCDD inhibited growth of LNCaP cells in the presence of DHT (data not shown). These results confirm that LNCaP cells are Ah-responsive and both TCDD and 6-MCDF inhibit LNCaP cell proliferation. The effects of TCDD on cell cycle progression was also determined in LNCaP cells treated with 1.0, 10 and 100 nM TCDD for 48 h followed by FACS analysis. The results show that TCDD induced a small but significant increase in the percentage of cells in G_0/G_1 and a decrease of cells in S phase, whereas minimal differences in distribution of cells in G_0/G_1 , S and G_2/M phases were observed in LNCaP cells treated with solvent (DMSO) or DHT (10 nM).

Inhibitory AhR-AR Crosstalk in LNCaP Cells Transfected with Androgen-responsive Constructs

Jana and coworkers (9) previously reported that TCDD inhibited testosterone-induced luciferase activity in LNCaP cells transfected with an androgen-responsive construct containing the mouse mammary tumor virus (MMTV) promoter. Inhibition of testosterone-induced PSA protein or mRNA by 100 nM TCDD was reported but not quantitated, and the magnitude of inhibition was minimal. Therefore, we further investigated inhibitory AhR-AR crosstalk in LNCaP cells transfected with pPB which

contains the -286 to +28 region of the androgen-responsive probasin gene promoter (Fig. 2A) (18). There was a > 13-fold increase in luciferase activity in LNCaP cells treated with 10 nM DHT and transfected with pPB and the induced response was significantly inhibited after cotreatment with DHT plus TCDD. Similar inhibitory responses were also observed using 2 μ M MCDF (Fig. 2A), whereas TCDD and MCDF alone did not significantly induce activity. Surprisingly, 10 nM E2 alone induces luciferase activity in LNCaP cells transfected with pPB, and the hormone-induced response is significantly decreased in cells cotreated with E2 plus TCDD or 6-MCDF (Fig. 2A).

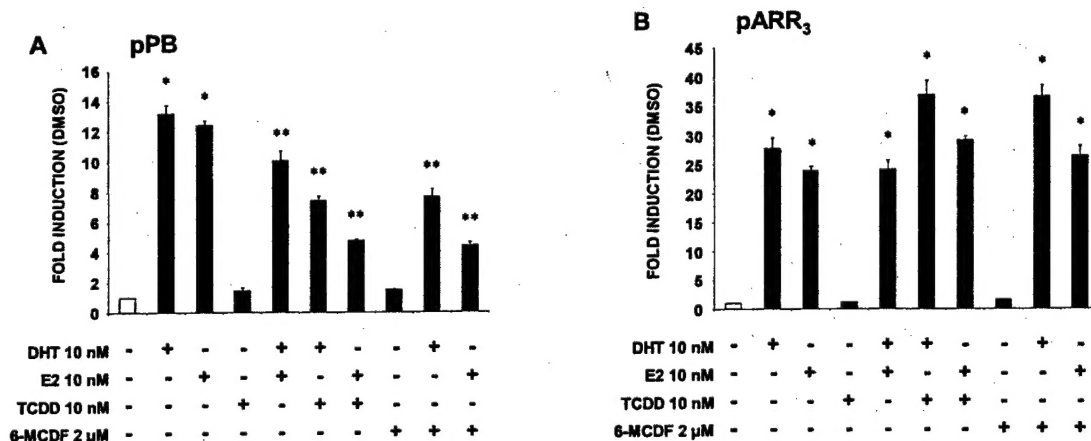


Figure 2. Inhibition of AR-dependent transactivation by TCDD and 6-MCDF. LNCaP cells were transfected with pPB [A] or pARR3 [B], treated with hormone or AhR agonist alone or in combination, and luciferase activity was determined as outlined in the Materials and Methods. Significant ($p < 0.05$) induction by compounds alone is indicated by an asterisk, and significant ($0 < 0.05$) inhibitory effects observed in cotreatment studies are also indicated (**). Results are expressed as means \pm SE for three replicate determinations for each treatment group.

The pARR₃ construct contains three tandem (3) copies of the probasin androgen response element, and was used to further investigate inhibitory AhR-AR crosstalk and the androgenic activity of E2. Ten nM DHT induced a > 27-fold increase in luciferase in LNCaP cells transfected with pARR₃; however, for this construct, cotreatment with DHT

plus MCDF or TCDD did not decrease DHT-induced activity (Fig. 2B). E2 (10 nM) also induced luciferase activity (> 24-fold) in cells transfected with pARR₃; however, in cells cotreated with E2 plus TCDD or MCDF, activity was not significantly decreased compared to that observed for E2 alone. These results confirmed that both DHT and E2 activated gene expression in cells transfected pPB or pARR₃; however, inhibitory effects of AhR agonists were observed only for the former construct.

The unexpectedly high AR agonist activity of E2 compared to DHT in LNCaP cells were further investigated in cells transfected with pPB and treated with hormones and antiandrogens or antiestrogens. Induction of luciferase activity by 10 nM DHT and E2 in LNCaP cells transfected with pPB was inhibited in cells cotreated with the hormone plus 10 μ M HPTE, an AR antagonist (Fig. 3A). However, in parallel studies, the "pure" antiestrogen ICI 182780 also significantly inhibited E2-induced activity, whereas only minimal inhibition was observed in LNCaP cells treated with DHT plus ICI 182780. In a parallel experiment in LNCaP cells transfected with pARR₃, both HPTE and ICI 182780 inhibited DHT and E2-induced luciferase activity (Fig. 3B), whereas 1 μ M flutamide, an AR antagonist, caused only minimal decreases in hormone-induced activity (Fig. 3C). HPTE is also an ER α agonist and ER β antagonist (19) and the results obtained for both HPTE and ICI 182780 suggest a possible role for ER β in mediating activation of pPB and pARR₃. However, previous studies show that endogenous ER β is insufficient for E2-induced transactivation in LNCaP cells transfected with pERE₃, a construct containing three tandem estrogen responsive elements (ERE₃) (20, 21), suggesting that activation of pPB or pARR₃ is ER β -independent. Therefore, in order to confirm the role of AR in mediating these responses, we further investigated hormone

activation of pPB and inhibitory AhR-AR crosstalk in ZR-75 cells which express minimal AR protein (22).

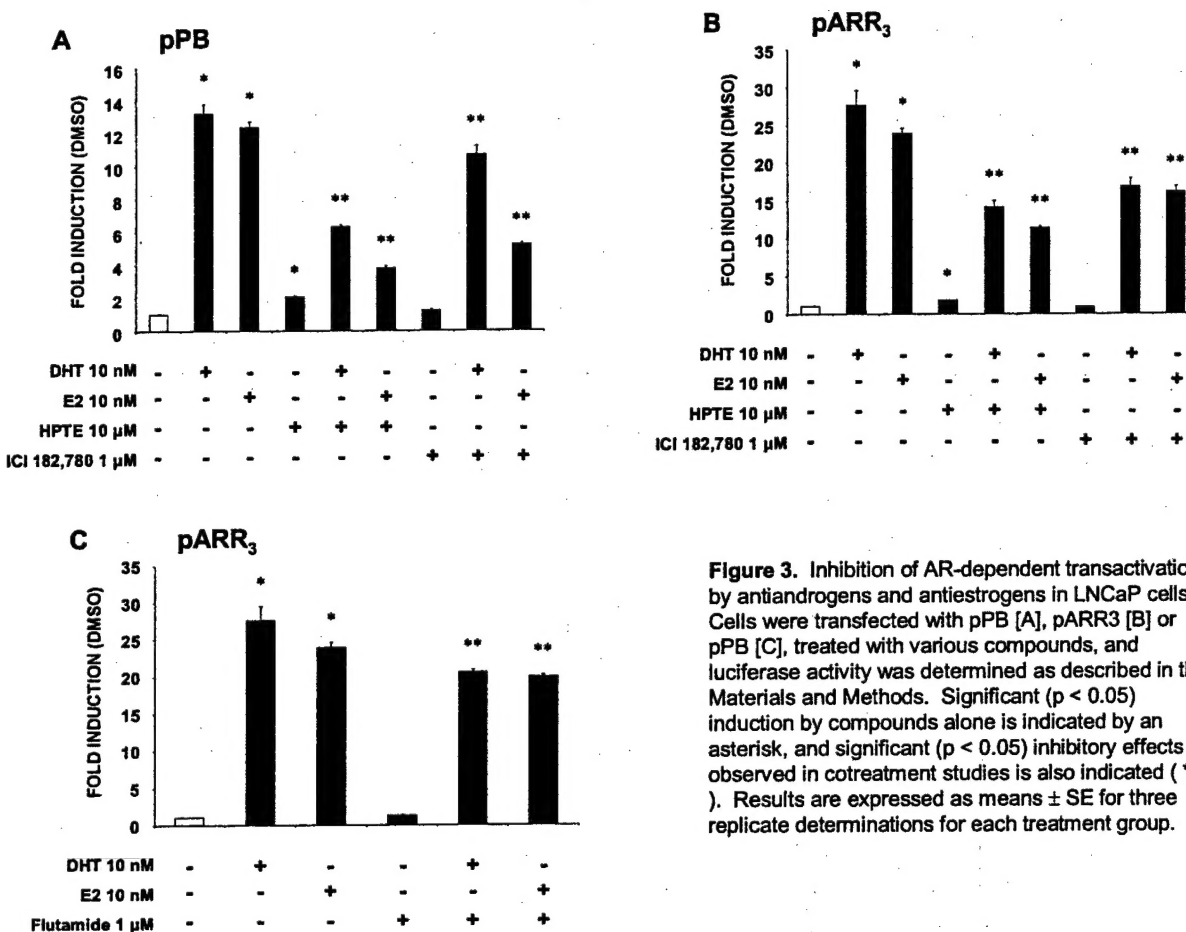


Figure 3. Inhibition of AR-dependent transactivation by antiandrogens and antiestrogens in LNCaP cells. Cells were transfected with pPB [A], pARR3 [B] or pPB [C], treated with various compounds, and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction by compounds alone is indicated by an asterisk, and significant ($p < 0.05$) inhibitory effects observed in cotreatment studies is also indicated (**). Results are expressed as means \pm SE for three replicate determinations for each treatment group.

Results in Figure 4A show that DHT, E2, TCDD and MCDF do not activate reporter gene activity in ZR-75 cells transfected with pPB alone; however, both DHT and E2 induced luciferase activity in cells cotransfected with pPB and hAR expression plasmid (Fig. 4B). Induction by E2 was significant but lower than observed for DHT in ZR-75 cells, and TCDD inhibited E2 but not DHT-induced activity in cells cotreated with hormone plus TCDD. Similar results were observed in duplicate experiments confirming that E2-dependent transactivation of pPB was AR-dependent. However, it was also evident that there were important differences between the interaction of TCDD

and DHT in LNCaP and ZR-75 cells since TCDD did not inhibit DHT-induced luciferase activity in the latter cell line. This suggests that inhibitory AhR-AR crosstalk is cell context-dependent for the pPB promoter.

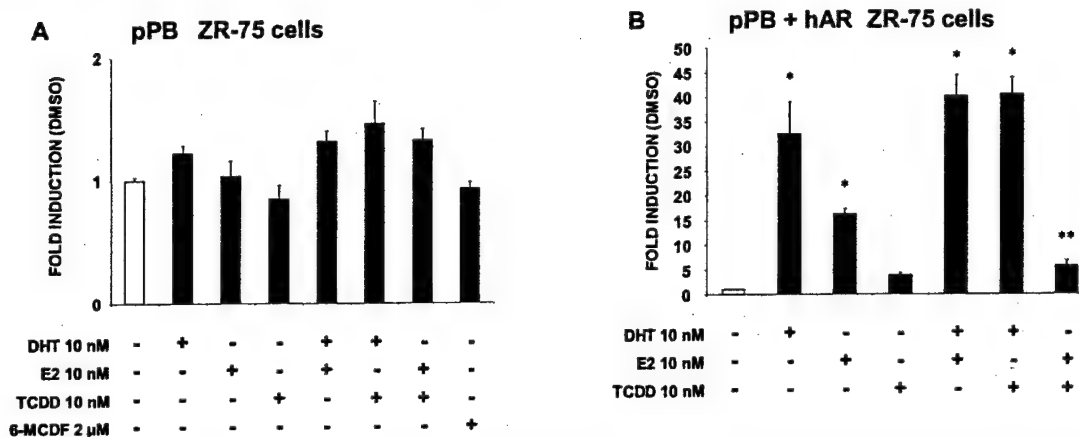


Figure 4. Inhibition of hormone-induced transactivation in ZR-75 breast cancer cells transfected with pPB. [A] Transfection with pPB alone. ZR-75 cells were transfected with pPB, treated with various compounds and luciferase activity was determined as described in the Materials and Methods. No significant induction was observed in any of the treatment groups. [B] Transfection with pPB and hAR. Cells were transfected and treated as described in [A] except that 500 ng of hAR expression plasmid was also transfected. Significant ($p < 0.05$) induction by compounds alone is indicated by an asterisk and significant inhibitory effects observed in cotreatment studies is also indicated (**). Results are expressed as means \pm SE for three replicate determinations for each treatment group.

Effects of Various Treatments on AR, Cyclin D1 and p27 Protein Levels in LNCaP

Cells

Levels of AR protein expression may influence androgen-responsiveness and inhibitory AhR-AR crosstalk, and the results in Figure 5A demonstrate levels of immunoreactive AR protein in LNCaP cells after various treatments. Preliminary studies in LNCaP and other cell lines indicated that any changes in AR expression were observed within 6 – 12 h after treatment (data not shown) and a 6 h time point was selected for this study. Treatment with 10 nM DHT, 10 nM E2 or DHT plus E2 resulted in a significant increase in AR levels. In contrast, 10 nM TCDD and 2 μ M 6-MCDF

alone did not significantly affect levels of AR protein; however, in combination with DHT, there was a significant decrease in AR levels compared to cells treated with DHT alone. TCDD in combination with E2 also decreased AR levels compared to those observed in cells treated with E2 alone. In contrast, levels of immunoreactive p27 protein were not significantly changed by any of the treatments (also observed in studies summarized in Fig. 1A), and served as a loading control for this experiment. In a separate study, the effects of the antiandrogen HPTE and the antiestrogen ICI 182780 alone and in combination with E2 or DHT on AR levels were also determined (Fig. 5B). Ten μ M HPTE alone did not affect AR levels in LNCaP cells, whereas ICI 182780 treatment increased AR levels compared to DMSO (solvent) treatment. Hormone (E2 or DHT)-

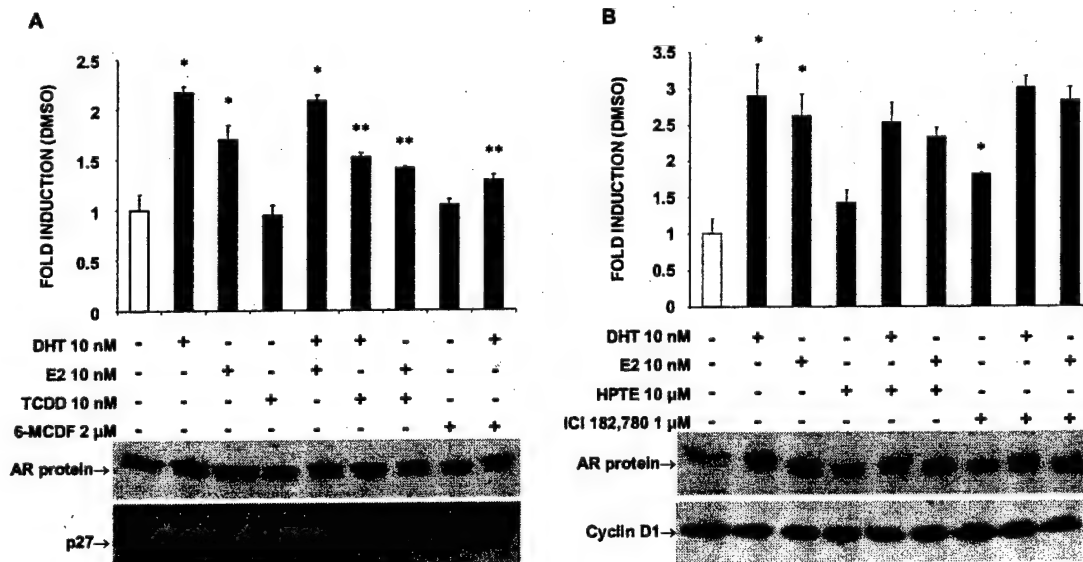


Figure 5. AR protein expression in LNCaP cells treated with hormones, AhR agonists, antiandrogens and antiestrogens. [A] AR protein expression in cells treated with hormones and AhR agonists. LNCaP cells were treated with DHT, E2, TCDD, 6-MCDF and their combinations for 6 h, and AR protein levels in whole cell lysates were determined by Western blot analysis as described in the Materials and Methods. p27 protein was also determined for this experiment; p27 was essentially unchanged in all of the treatment groups and serves as a loading control for this experiment. [B] AR protein expression in cells treated with hormones, antiandrogens and antiestrogens. AR protein levels were determined essentially as described in [A] and blots were stripped and reprobed with cyclin D1 antibodies. Cyclin D1 protein was unchanged in this experiment and serves as a loading control. For studies illustrated in [A] and [B], significant ($p < 0.05$) increases in AR protein levels by individual compounds are indicated by an asterisk, and significant ($p < 0.05$) decreases in the cotreatment groups are also indicated (**). Results are expressed as means \pm SE for three replicate determinations for each treatment group.

induced upregulation of AR protein was not decreased cotreatment with HPTE or ICI 182780. Cyclin D1 protein was not significantly changed in this study and served as a loading control (also see Fig. 1A). These data demonstrate that various treatments differentially modulate AR protein levels in LNCaP cells, and current studies are focused on the influence of ligand-induced changes in AR expression and the magnitude of hormone-induced transactivation.

KEY RESEARCH ACCOMPLISHMENTS

- Growth of LNCaP prostate cancer cells is inhibited by TCDD and 6-MCDF.
- Inhibitory AhR-AR crosstalk was observed in LNCaP cells transfected with a construct (pPB) containing the androgen-responsive probasin promoter (-288 to +28).
- E2 was a potent androgen in LNCaP cells.
- E2 and DHT stabilized AR protein levels.
- AhR agonists partially inhibited stabilization of AR protein.

REPORTABLE OUTCOMES

Morrow, D., Qin, C., Smith III, R. and Safe, S. Aryl hydrocarbon receptor-mediated inhibition of LNCaP prostate cancer cell growth and hormone-induced transactivation. *J. Steroid Biochem. Mol. Biol.* in press, 2004.

Morrow, D. and Safe, S. Aryl hydrocarbon receptor agonists inhibit hormone-induced transactivation in prostate cancer cells. Society of Toxicology Annual Meeting, Salt Lake City, UT, 2003.

CONCLUSIONS

LNCaP prostate cancer cells express the aryl hydrocarbon receptor (AhR), and treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces CYP1A1 protein and an Ah-responsive reporter gene. Similar results were obtained with the selective AhR modulator 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF); however, TCDD but not 6-MCDF induced degradation of the AhR protein. TCDD and 6-MCDF inhibited growth of LNCaP cells, and inhibitory AhR-androgen receptor (AR) crosstalk was investigated in cells transfected with constructs containing the androgen-responsive probasin promoter (-288 to +28) (pPB) or three copies of the -244 to -96 region of this promoter (pARR₃). Ten nM dihydrotestosterone (DHT) and 17 β -estradiol (E2) induced transactivation in LNCaP cells transfected with pPB or pARR₃; however, inhibitory AhR-AR crosstalk was observed only with the latter construct. 6-MCDF and TCDD did not inhibit DHT- or E2-induced transactivation in ZR-75 human breast cancer cells, indicating that these interactions were promoter and cell context-dependent. Both E2 and DHT stabilized AR protein in LNCaP cells; however, cotreatment with TCDD or 6-MCDF decreased AR protein levels. These results indicate that inhibitory AhR-AR crosstalk in prostate cancer cells is complex and for some responses, AR protein stability may play a role.

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APPENDIX

1. Morrow, D., Qin, C., Smith III, R. and Safe, S. Aryl hydrocarbon receptor-mediated inhibition of LNCaP prostate cancer cell growth and hormone-induced transactivation. *J. Steroid Biochem. Mol. Biol.* in press, 2004.
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Aryl hydrocarbon receptor-mediated inhibition of LNCaP prostate cancer cell growth and hormone-induced transactivation

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Abstract

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Keywords: Ah receptor; Androgen receptor; Inhibitory crosstalk; LNCaP cells

1. Introduction

Prostate cancer is the most commonly diagnosed cancer in North American men and it is estimated that there are over 300,000 newly diagnosed cases each year [1,2]. The incidence and mortality rates from prostate cancer are increasing and this is due, in part, to an increasingly aging population and the higher incidence of this disease in older men [3,4]. Prostate cancer therapy is dependent on the stage of the tumor and androgen receptor (AR) expression. Early stage androgen-responsive prostate cancers can be treated by castration or with antiandrogens or drugs that block androgen-induced responses including steroidal antiandrogens (cyproterone), luteinizing hormone releasing hormone (LHRH) analogs, nonsteroidal antiandrogens (flutamide, nilutamide, bicalutamide), and the potent estrogenic drug diethylstilbestrol (reviewed in [5–8]). In addition, there are several novel strategies for treatment of prostate cancer and other tumor-types and these include targeting of critical

genes involved in tumor cell growth and metastasis (e.g., antiangiogenic drugs, antisense therapy) [9–13]. Ligands for nuclear receptors (NR) are also being developed for treatment of prostate cancer through inhibitory NR-AR crosstalk that involves various compounds that bind the retinoid acid/X-receptors (retinoids), vitamin D receptor (calcitriol), and peroxisome proliferator activate receptor γ (thiazolidinedione-derived drugs) [14–26]. A recent study in androgen-responsive LNCaP prostate cancer cells showed that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a ligand for the aryl hydrocarbon receptor (AhR), inhibited testosterone-induced cell proliferation and gene/reporter gene expression [27].

The AhR was initially identified as the intracellular receptor that bound TCDD and related toxic halogenated aromatic hydrocarbons [28,29]; however, more recent studies show that chemoprotective phytochemicals and other structurally-diverse chemicals also interact with this receptor [30]. There is also evidence that the AhR is a potential target for drug development since long-term feeding studies with TCDD in female Sprague–Dawley rats showed that development of several age-dependent cancers including

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17 β -estradiol (E2)-dependent mammary and uterine tumors were inhibited [31]. Subsequent studies have demonstrated inhibitory AhR-ER crosstalk in the rodent uterus, rodent mammary tumors, breast and endometrial cancer cells [28,32–34]. In addition to the reported growth inhibitory effects of TCDD in prostate cancer cells, recent studies show that AhR agonists also inhibit growth of pancreatic cancer cells [35].

Research in this laboratory is focused on development of selective AhR modulators (SAhRMs) that exhibit tissue-specific AhR agonist or antagonist activity [36,37]. Alternate substituted (1,3,6,8- or 2,4,6,8-) alkyl polychlorinated dibenzofurans, typified by 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF), are relatively non-toxic and inhibit prototypical AhR-mediated toxic responses in rodent models (i.e., AhR antagonists) but exhibit selective AhR-dependent antiestrogenic and antitumorigenic activities in mammary tumor models [38–46]. 6-MCDF also inhibits growth of some ER-negative breast cancer [47] and pancreatic cancer cells [35]. This paper describes inhibition of LNCaP prostate cancer cell growth by TCDD and 6-MCDF, and both compounds also inhibit E2- and androgen-induced transactivation in LNCaP cells transfected with an androgen-responsive construct containing probasin gene promoter inserts.

2. Materials and methods

2.1. Chemicals, biochemicals, and plasmids

Fetal bovine serum (FBS) was obtained from Summit Biotechnology (Fort Collins, CO). RPMI 1640 medium, phenol-free Dulbecco's modified Eagle's medium/F-12 medium, phosphate-buffered saline, 100 \times antibiotic/antimycotic solution, *N*-[2-hydroxyethyl]piperazine-*N'*[2-ethanesulfonic acid] (HEPES), 17 β -estradiol (E2), and dihydrotestosterone (DHT) were purchased from Sigma; 5 \times reporter lysis buffer and luciferin were purchased from Promega (Madison, WI). Reagents for β -galactosidase analysis were purchased from Applied Biosystems (Foster City, CA). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) were synthesized in this laboratory. Forty percent polyacrylamide was obtained from National Diagnostics (Atlanta, GA). PB-luc and ARR₃TK-luc [48] constructs were the generous gifts of Dr. Robert J. Matusik (Vanderbilt University Medical Center, Nashville, TN). Human AR (hAR) expression plasmid [49] was kindly provided jointly by Drs. Kerry L. Burnstein (University of Miami School of Medicine) and Michael J. McPhaul (U.T. Southwestern Medical School, Dallas, TX). The pcDNA3.1- β -gal plasmid was obtained from Invitrogen (Carlsbad, CA). The pDRE₃-luciferase reporter plasmid was constructed in this laboratory and contains three tandem consensus dioxin response elements (DRE) (TCT TCT CAC GCA ACT CCG A—a single DRE sequence). All other chemicals and biochemicals were the

highest quality available from commercial sources. Scheduled substances were procured, stored, and disposed in compliance with relevant federal and state laws.

2.2. Transient transfection assays

ZR-75 human breast cancer and LNCaP human prostate cancer cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 medium supplemented with 10% FBS, 1% antibiotic/antimycotic solution, 1.5 g/l sodium bicarbonate, and 10 mM HEPES, final pH of 7.4. Cells were seeded at 2.75×10^5 per 22-mm well in DME-F12 without phenol red, supplemented with 2.5% charcoal-stripped FBS. After 24 h, cells were transfected using Lipofectamine and Plus reagents (Invitrogen) according to manufacturer's instructions. LNCaP and ZR-75 cells were transfected with 500 ng per well of either reporter plasmid, and 250 ng per well of pcDNA3.1- β -gal (Invitrogen) as the internal control. In addition, ZR-75 cells were transfected with 500 ng hAR. Twenty-four hours after treatment, cells were harvested by scraping with 200 μ l per well of reporter lysis buffer. Lysates were centrifuged at $40,000 \times g$ and luciferase and β -galactosidase activity was assayed with 30 μ l of the supernatant extract per sample using a Lumicount luminometer (Perkin-Elmer, Boston, MA). Luciferase activity was normalized to β -galactosidase activity for each transfection well. Results of transfection experiments are expressed as means \pm S.E. compared to the DMSO control group, which is set at 1.

2.3. Cell proliferation assay

After trypsinization and low-speed centrifugation, LNCaP cells were resuspended and counted using a Coulter cell counter (Beckman Coulter, Fullerton, CA). Cells were seeded at a density of 5×10^4 /35-mm well using DME-F12 without phenol red, supplemented with 2.5% charcoal-stripped FBS. Twenty-four hours after seeding, initial treatment was applied and then subsequently reapplied with fresh medium every two days until harvesting by trypsinization. Cells were counted after harvesting using a Coulter counter.

2.4. Fluorescence activated cell sorting analysis

Cells were analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA) flow cytometer, equipped with a 15 mW air-cooled argon laser, using CellQuest (Becton Dickinson) acquisition software. Propidium iodide (PI) fluorescence was collected through a 585/42-nm bandpass filter, and list mode data were acquired on a minimum of 12,000 single cells defined by a dot plot of PI-width versus PI-area. Data analysis was performed in ModFit LT (Verity Software House, Topsham, ME) using PI-width versus PI-area to exclude cell

Previous studies reported that the AhR and Arnt mRNA are expressed in LNCaP cells and Ah-responsiveness was confirmed by induction of CYP1A1 mRNA and CYP1A1-dependent EROD activity by TCDD [27]. Results illustrated in Fig. 1A show that 10 nM TCDD, 2 and 5 μ M 6-MCDF induce CYP1A1 protein in LNCaP cells, and this is consistent with previous reports showing that 10 nM TCDD induces CYP1A1-dependent EROD activity [27]. Western blot analysis also confirmed expression of both AhR and Arnt proteins, and treatment with TCDD but

LNCaP cells

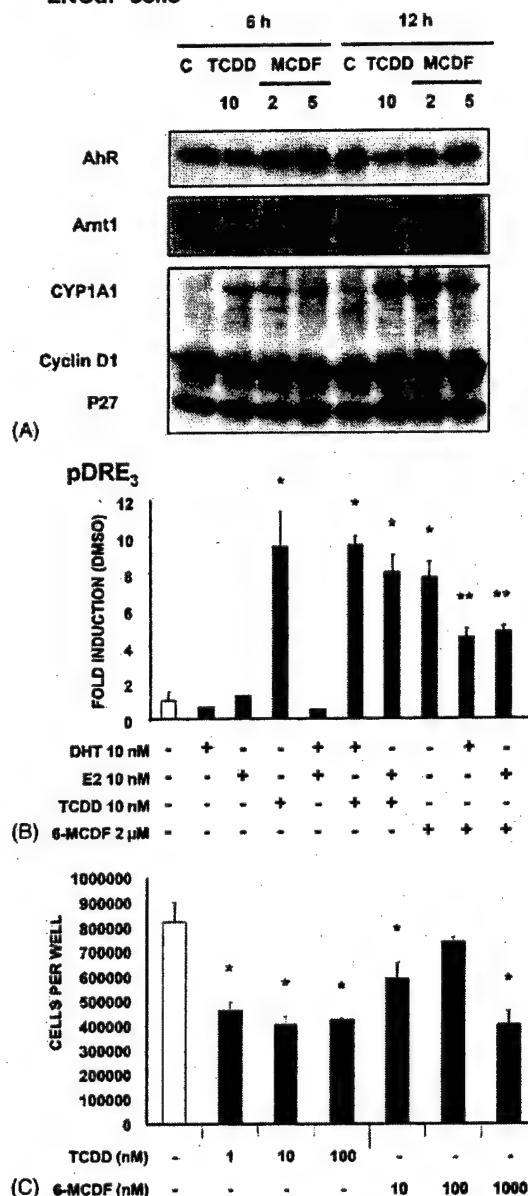


Fig. 1. Ligand-dependent AhR activation and growth inhibition in LNCaP cells. (A) Induction of CYP1A1 protein. LNCaP cells were treated with DMSO (C), 10nM TCDD, 2 or 5 μ M 6-MCDF for 6 or 12 h, and whole cell lysates were analyzed by Western blot analysis as described in Section 2. Antibodies were used to detect the AhR, Arnt, CYP1A1, cyclin D1 and p27 proteins. (B) Activation of pDRE₃. LNCaP cells were transfected with pDRE₃, treated with various compounds and luciferase activity was determined as described in Section 2. Significant induction ($P < 0.05$) is indicated with an asterisk and inhibition of TCDD- or 6-MCDF-induced activity is also indicated (**). (C) Inhibition of LNCaP cell growth by TCDD and 6-MCDF. Cells were cultured for six days, treated with different concentrations of TCDD or 6-MCDF, and cell numbers were determined as described in Section 2. Significant ($P < 0.05$) growth inhibition is indicated by an asterisk. All results are presented as means \pm S.E. for three replicate determinations for each treatment group. Growth inhibition in some of the groups was observed after 2 to 4 days.

Table 1

Effects of TCDD on cell cycle progression in LNCaP prostate cancer cells^a

Treatment	Percent distribution		
	G ₀ /G ₁	G ₂ /M	S
DMSO	70.300 ± 1.779	10.973 ± 0.544	18.7 ± 1.258
TCDD (10 ⁻⁹ M)	74.300 ± 0.751*	10.633 ± 0.376	14.7 ± 0.520*
TCDD (10 ⁻⁸ M)	75.367 ± 0.636*	10.300 ± 0.153	14.3 ± 0.666*
TCDD (10 ⁻⁷ M)	77.500 ± 0.451*	8.943 ± 0.471	13.567 ± 0.176*
DHT (10 ⁻⁸ M)	73.400 ± 1.179	9.433 ± 1.011	17.167 ± 0.296

^a LNCaP cells were treated as indicated for 48 h and the percentage distribution of cells in G₀/G₁, G₂/M, and S phases were determined by FACS analysis as described in Section 2. Significant ($p < 0.05$) effect compared to DMSO are indicated by an asterisk.

not 6-MCDF decreased expression of the AhR. Expression of other proteins including Sp1, cyclin D1 and p27 were unaffected by the treatments and serve as loading controls. Results illustrated in Fig. 1B also show that treatment of LNCaP cells with 10 nM TCDD induced luciferase activity >9-fold compared to solvent control (DMSO) in cells transfected with pDRE₃. In contrast, 10 nM DHT, 10 nM E2 and E2 plus DHT did not significantly induce activity, and neither DHT or E2 in combination with TCDD affected induced activity. 6-MCDF (2 μM), a prototypical SAhRM, also induced luciferase activity (>7-fold), and this was consistent with the induction of CYP1A1 by 6-MCDF. 6-MCDF is a much less potent agonist for activation of CYP1A1 or DRE-dependent activities in breast cancer cells [42]. Both E2 and DHT in combination with 6-MCDF significantly inhibited 6-MCDF-induced activity, whereas in cells treated with TCDD in combination with E2 or DHT, inhibitory interactions were not observed.

The comparative effects of TCDD and 6-MCDF on growth of LNCaP cells were also determined in cells treated with solvent control and different concentrations of the AhR agonists for 6 days. The results show that TCDD (1–100 nM) significantly inhibited proliferation of LNCaP cells, and growth inhibition was also observed for 6-MCDF (Fig. 1B). Both compounds inhibited ≥50% cell growth at one or more concentrations. Similar experiments were also carried out with 6-MCDF and TCDD in LNCaP cells also treated with different concentrations of DHT (up to 10 nM). Hormone-induced cell growth was not observed; however, both 6-MCDF and TCDD inhibited growth of LNCaP cells in the presence of DHT (data not shown). These results confirm that LNCaP cells are Ah-responsive and both TCDD and 6-MCDF inhibit LNCaP cell proliferation. The effects of TCDD on cell cycle progression was also determined in LNCaP cells treated with 1.0, 10 and 100 nM TCDD for 48 h followed by FACS analysis (Table 1). The results show that TCDD induced a small but significant increase in the percentage of cells in G₀/G₁ and a decrease of cells in S phase, whereas solvent (DMSO) and DHT (10 nM) exhibited minimal differences.

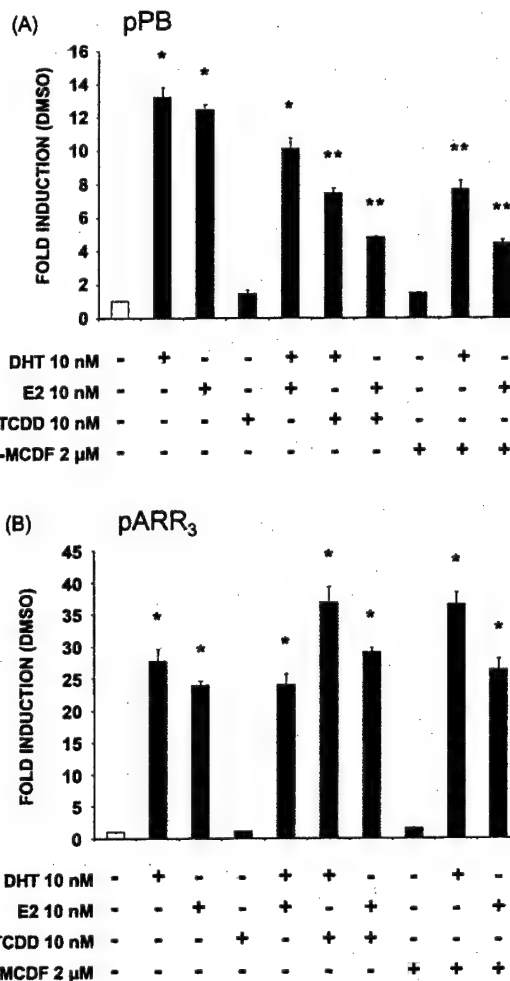


Fig. 2. Inhibition of AR-dependent transactivation by TCDD and 6-MCDF. LNCaP cells were transfected with pPB (A) or pARR₃ (B), treated with hormone or AhR agonist alone or in combination, and luciferase activity was determined as outlined in Section 2. Significant ($P < 0.05$) induction by compounds alone is indicated by an asterisk, and significant ($0 < 0.05$) inhibitory effects observed in cotreatment studies are also indicated (**). Results are expressed as means ± S.E. for three replicate determinations for each treatment group.

3.2. Inhibitory AhR-AR crosstalk in LNCaP cells transfected with androgen-responsive constructs

Jana et al. [27] previously reported that TCDD inhibited testosterone-induced luciferase activity in LNCaP cells transfected with an androgen-responsive construct containing the mouse mammary tumor virus (MMTV) promoter. Inhibition of testosterone-induced PSA protein or mRNA by 100 nM TCDD was reported but not quantitated, and the magnitude of inhibition was minimal. Therefore, we further investigated inhibitory AhR-AR crosstalk in LNCaP cells transfected with pPB which contains the -286 to +28 region of the androgen-responsive probasin gene promoter (Fig. 2A). There was a >13-fold increase in luciferase

activity in LNCaP cells treated with 10 nM DHT and transfected with pPB and the induced response was significantly inhibited after cotreatment with DHT plus TCDD. Similar inhibitory responses were also observed using 2 μ M MCDF (Fig. 2A), whereas TCDD and MCDF alone did not significantly induce activity. Surprisingly, 10 nM E2 alone induces luciferase activity in LNCaP cells transfected with pPB, and the hormone-induced response is significantly decreased in cells cotreated with E2 plus TCDD or 6-MCDF (Fig. 2A).

The pARR₃ construct contains three tandem (3) copies of the probasin androgen response element, and was used to further investigate inhibitory AhR-AR crosstalk and the androgenic activity of E2. Ten nanomolar DHT induced a >27-fold increase in luciferase in LNCaP cells transfected with pARR₃; however, for this construct, cotreatment with DHT plus MCDF or TCDD did not decrease DHT-induced activity (Fig. 2B). E2 (10 nM) also induced luciferase activity (>24-fold) in cells transfected with pARR₃; however, in cells cotreated with E2 plus TCDD or MCDF, activity was not significantly decreased compared to that observed for E2 alone. These results confirmed that both DHT and E2 activated gene expression in cells transfected pPB or pARR₃; however, inhibitory effects of AhR agonists were observed only for the former construct.

The unexpectedly high AR agonist activity of E2 compared to DHT in LNCaP cells were further investigated in cells transfected with pPB and treated with hormones and antiandrogens or antiestrogens. Induction of luciferase activity by 10 nM DHT and E2 in LNCaP cells transfected with pPB was inhibited in cells cotreated with the hormone plus 10 μ M HPTE, an AR antagonist (Fig. 3A). However, in parallel studies, the "pure" antiestrogen ICI 182780 also significantly inhibited E2-induced activity, whereas only minimal inhibition was observed in LNCaP cells treated with DHT plus ICI 182780. In a parallel experiment in LNCaP cells transfected with pARR₃, both HPTE and ICI 182780 inhibited DHT and E2-induced luciferase activity (Fig. 3B), whereas 1 μ M flutamide, an AR antagonist, caused only minimal decreases in hormone-induced activity (Fig. 3C). HPTE is also an ER α agonist and ER β antagonist [50] and the results obtained for both HPTE and ICI 182780 suggest a possible role for ER β in mediating activation of pPB and pARR₃. However, previous studies show that endogenous ER β is insufficient for E2-induced transactivation in LNCaP cells transfected with pERE₃, a construct containing three tandem estrogen responsive elements (ERE₃) [51,52], suggesting that activation of pPB or pARR₃ is ER β -independent. Therefore, in order to confirm the role of AR in mediating these responses, we further investigated hormone activation of pPB and inhibitory AhR-AR crosstalk in ZR-75 cells which express minimal AR protein [53]. Results in Fig. 4A show that DHT, E2, TCDD and MCDF do not activate reporter gene activity in ZR-75 cells transfected with pPB alone; however, both DHT and E2 induced luciferase activity in cells cotransfected with pPB and hAR expression plasmid (Fig. 4B). Induction by E2 was significant but

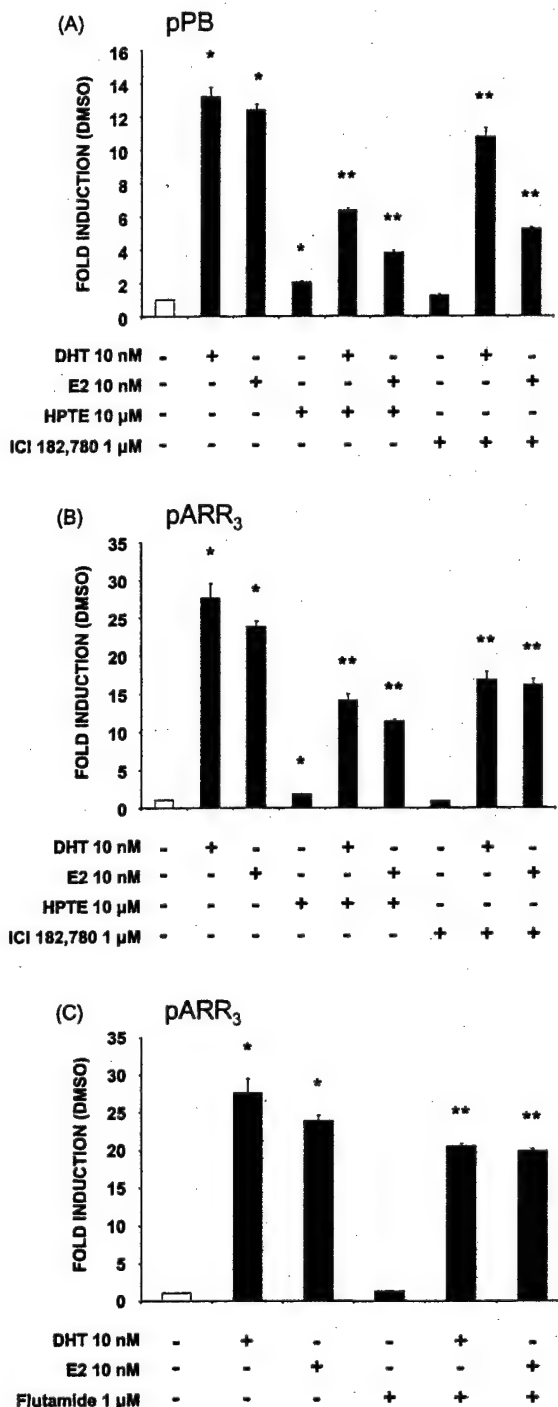


Fig. 3. Inhibition of AR-dependent transactivation by antiandrogens and antiestrogens in LNCaP cells. Cells were transfected with pPB (A), pARR₃ (B) or pPB (C), treated with various compounds, and luciferase activity was determined as described in Section 2. Significant ($P < 0.05$) induction by compounds alone is indicated by an asterisk, and significant ($P < 0.05$) inhibitory effects observed in cotreatment studies is also indicated (**). Results are expressed as means \pm S.E. for three replicate determinations for each treatment group.

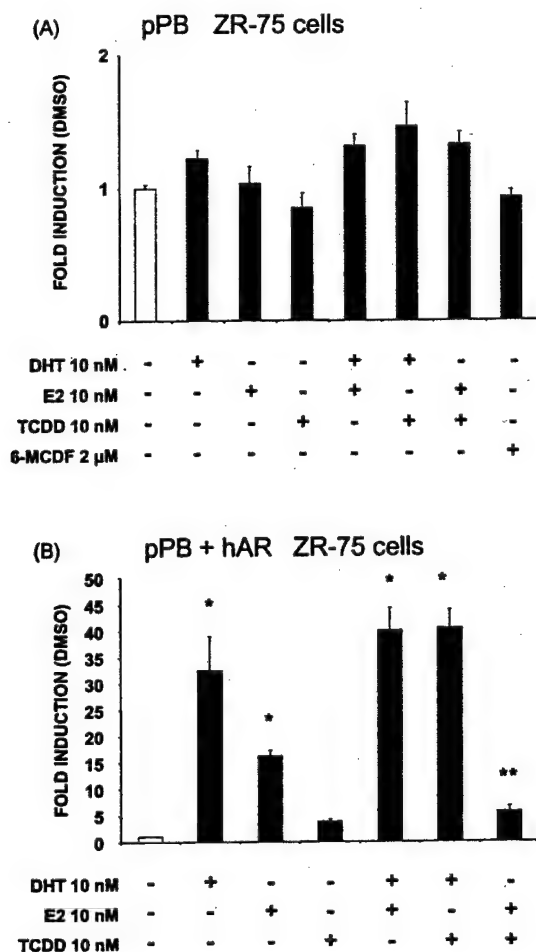


Fig. 4. Inhibition of hormone-induced transactivation in ZR-75 breast cancer cells transfected with pPB. (A) Transfection with pPB alone. ZR-75 cells were transfected with pPB, treated with various compounds and luciferase activity was determined as described in the Materials and Methods. No significant induction was observed in any of the treatment groups. (B) Transfection with pPB and hAR. Cells were transfected and treated as described in (A) except that 500 ng of hAR expression plasmid was also transfected. Significant ($P < 0.05$) induction by compounds alone is indicated by an asterisk and significant inhibitory effects observed in cotreatment studies is also indicated (**). Results are expressed as means \pm S.E. for three replicate determinations for each treatment group.

lower than observed for DHT in ZR-75 cells, and TCDD inhibited E2 but not DHT-induced activity in cells cotreated with hormone plus TCDD. Similar results were observed in duplicate experiments confirming that E2-dependent transactivation of pPB was AR-dependent. However, it was also evident that there were important differences between the interaction of TCDD and DHT in LNCaP and ZR-75 cells since TCDD did not inhibit DHT-induced luciferase activity in the latter cell line. This suggests that inhibitory AhR-AR crosstalk is cell context-dependent for the pPB promoter.

3.3. Effects of various treatments on AR, cyclin D1 and p27 protein levels in LNCaP cells

Levels of AR protein expression may influence androgen-responsiveness and inhibitory AhR-AR crosstalk, and the results in Fig. 5A demonstrate levels of immunoreactive AR protein in LNCaP cells after various treatments. Preliminary studies in LNCaP and other cell lines indicated that any changes in AR expression were observed within 6–12 h after treatment (data not shown) and a 6 h time point was selected for this study. Treatment with 10 nM DHT, 10 nM E2 or DHT plus E2 resulted in a significant increase in AR levels. In contrast, 10 nM TCDD and 2 μ M 6-MCDF alone did not significantly affect levels of AR protein; however, in combination with DHT, there was a significant decrease in AR levels compared to cells treated with DHT alone. TCDD in combination with E2 also decreased AR levels compared to those observed in cells treated with E2 alone. In contrast, levels of immunoreactive p27 protein were not significantly changed by any of the treatments (also observed in studies summarized in Fig. 1A), and served as a loading control for this experiment. In a separate study, the effects of the antiandrogen HPTE and the antiestrogen ICI 182780 alone and in combination with E2 or DHT on AR levels were also determined (Fig. 5B). Ten micromolar HPTE alone did not affect AR levels in LNCaP cells, whereas ICI 182780 treatment increased AR levels compared to DMSO (solvent) treatment. Hormone (E2 or DHT)-induced upregulation of AR protein was not decreased cotreatment with HPTE or ICI 182780. Cyclin D1 protein was not significantly changed in this study and served as a loading control (also see Fig. 1A). These data demonstrate that various treatments differentially modulate AR protein levels in LNCaP cells, and current studies are focused on the influence of ligand-induced changes in AR expression and the magnitude of hormone-induced transactivation.

4. Discussion

The AhR was initially characterized by its high affinity, low capacity binding to TCDD and related toxic halogenated aromatic hydrocarbons [54]. However, recent studies have demonstrated that the AhR also interacts with structurally diverse synthetic chemicals, drugs, endogenous biochemicals, and phytochemicals [30,55–57]. Moreover, many of these compounds such as synthetic retinoids, bioflavonoids, indole-3-carbinol and diindolylmethane (DIM) exhibit chemoprotective and anticarcinogenic properties in laboratory animal studies [58–63]. 6-MCDF is an example of a relatively non-toxic synthetic AhR agonist/antagonist that inhibits several TCDD-induced toxic responses including cleft palate, immunotoxicity and porphyria in mice and CYP1A1 in both in vivo and in vitro models [38–41]. However, 6-MCDF exhibits selective AhR agonist activity as an antiestrogen and inhibits E2-dependent mammary tumor

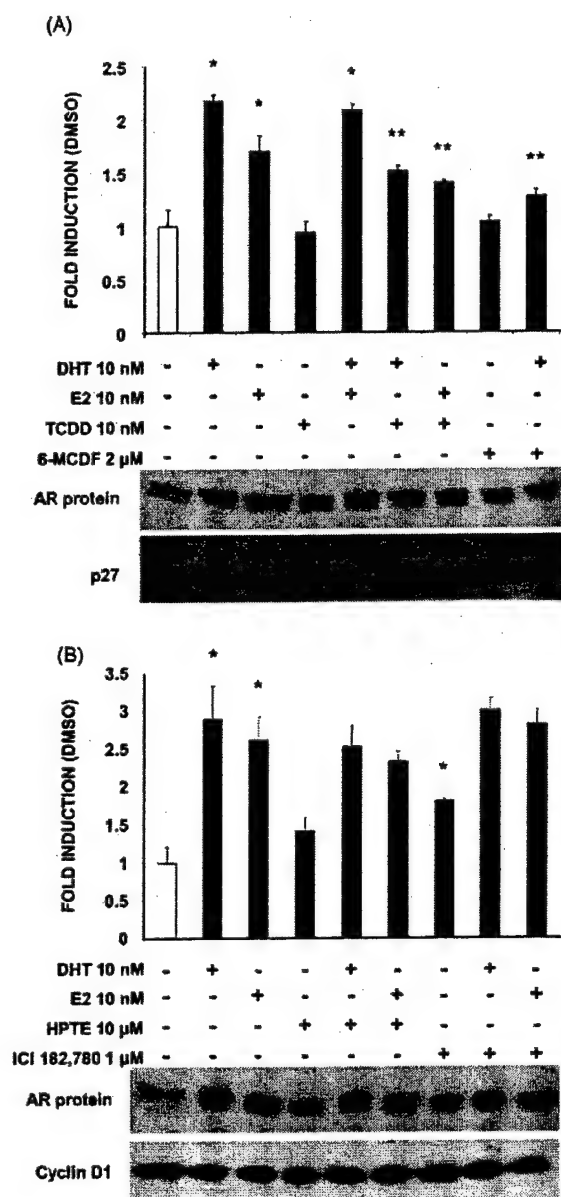


Fig. 5. AR protein expression in LNCaP cells treated with hormones, AhR agonists, antiandrogens and antiestrogens. (A) AR protein expression in cells treated with hormones and AhR agonists. LNCaP cells were treated with DHT, E2, TCDD, 6-MCDF and their combinations for 6 h, and AR protein levels in whole cell lysates were determined by Western blot analysis as described in Section 2. p27 protein was also determined for this experiment; p27 was essentially unchanged in all of the treatment groups and serves as a loading control for this experiment. (B) AR protein expression in cells treated with hormones, antiandrogens and antiestrogens. AR protein levels were determined essentially as described in (A) and blots were stripped and reprobed with cyclin D1 antibodies. Cyclin D1 protein was unchanged in this experiment and serves as a loading control. For studies illustrated in (A) and (B), significant ($P < 0.05$) increases in AR protein levels by individual compounds are indicated by an asterisk, and significant ($P < 0.05$) decreases in the cotreatment groups are also indicated (**). Results are expressed as means \pm S.E. for three replicate determinations for each treatment group.

growth (in vivo) and breast/endometrial cancer cell growth [42–46].

Recent studies show that 6-MCDF also inhibits E2-independent pancreatic cancer cell growth [35], and results of this study show that both TCDD and 6-MCDF inhibit growth of LNCaP cells (Fig. 1C), decrease the percentage of cells in S phase, and increase the percentage in G₀/G₁ (Table 1). Although the percentage of cells in G₀/G₁ and S phase are significantly affected by TCDD, the changes are relatively small suggesting that modulation of cell cycle genes by TCDD may not be a critical pathway for growth inhibition. Treatment of LNCaP cells with up to 10 nM DHT did not increase cell growth (data not shown) or G₀/G₁ \rightarrow S phase progression (Table 1); however, TCDD and 6-MCDF also inhibited LNCaP cell growth in the presence of DHT (data not shown). In addition, 6-MCDF and TCDD did not affect expression of cyclin D1 or p27 (Figs. 1A and 5), and only minimal expression of p21 was observed in the treatment groups (data not shown). Current studies are further investigating the mechanisms of LNCaP cell growth inhibition by AhR agonists.

Jana and coworkers [27,64] have reported inhibitory AhR-AR crosstalk in LNCaP cells and showed that 10 or 100 nM testosterone inhibited EROD activity induced by 100 nM TCDD and that TCDD inhibited testosterone-induced activation of an androgen-responsive construct containing the MMTV promoter. Results in Fig. 1A and B confirm the Ah-responsiveness of LNCaP cells. Both the AhR and Arnt proteins are expressed in LNCaP cells, and CYP1A1 protein is induced by TCDD and 6-MCDF. The induction of CYP1A1 by 6-MCDF was surprising since previous studies in breast cancer cells, rodent mammary tumors, and rodent liver show that this compound only weakly induces CYP1A1, and in cotreatment studies (TCDD + 6-MCDF), 6-MCDF inhibits induction of CYP1A1 by TCDD [38–45]. Treatment of LNCaP cells with TCDD resulted in decreased AhR protein expression, and this is consistent with studies in other cell lines where TCDD activates proteasome-dependent degradation of the AhR [45,65–67]. In contrast, 2 or 5 μ M 6-MCDF did not decrease AhR protein levels, and differences between the effects of TCDD and 6-MCDF correlated with reports showing that interactions of these compounds with the AhR induce different conformation of the bound receptor complex [68]. Thus, although TCDD and 6-MCDF induce similar responses, there are differences in their mode of action. TCDD and 6-MCDF also induced luciferase activity in cells transfected with pDRE₃ (Fig. 1B). Hormone-dependent decreases in TCDD-induced activity were not observed, whereas both E2 and DHT inhibited luciferase activity induced by 6-MCDF. This is consistent with a potential squelching mechanism where the AR and AhR compete for common cofactors, and inhibitory AR-AhR crosstalk is observed only with a less potent AhR agonists.

Inhibitory AhR-AR crosstalk was investigated using two related androgen-responsive constructs, pPB and pARR₃. pPB contains the -286 to +28 region of the probasin gene

promoter and the more androgen-responsive pARR₃ construct contains three copies of the –244 to –96 region of the rat probasin gene promoter [48,69]. The results in Fig. 2 demonstrate that both E2 and DHT induce luciferase activity in LNCaP cells transfected with pPB and pARR₃. Significant inhibition of DHT- and E2-induced activity by 10 nM TCDD or 6-MCDF was observed in cells transfected with pPB but not pARR₃. The inhibitory AhR-AR crosstalk in LNCaP cells transfected with pPB complements results of previous studies using a construct with a human PSA gene promoter insert [27]. The results obtained for pARR₃ and pPB also demonstrate that inhibitory crosstalk is promoter specific; differences may be due to promoter flanking sequences within the PB promoter that are not present in the pARR₃ construct and this is currently being investigated.

Both E2 and DHT activated pPB and pARR₃ in LNCaP cells, and patterns of inhibition by antiandrogens and antiestrogens were comparable (Figs. 2 and 3). Moreover, activation of pPB in AR-negative AR-75 cells [70] required cotransfection with AR expression plasmid (Fig. 4). These data are consistent with previous results showing that the mutant AR (Thr877Ala) expressed in LNCaP cells exhibits increased responsiveness to E2 [71,72].

Studies in this laboratory have demonstrated that inhibitory AhR-ER α crosstalk is associated with proteasome-dependent downregulation of ER α that results in limiting levels of this receptor [70]. Moreover, a recent report also showed that inhibition of androgen-induced transactivation by genistein in LNCaP cells was associated with genistein-induced downregulation of the AR [71]. We therefore investigated ligand-dependent changes in AR protein levels in LNCaP and other prostate and breast cancer cells, and preliminary time-course studies showed that AR levels stabilized within 6–24 h after treatment with hormones and/or their inhibitors. Results in Fig. 5 illustrate ligand-dependent changes in AR protein levels after treatment with hormones, AhR agonists, antiandrogen/antiestrogen compounds and their combinations. DHT increased levels of AR in LNCaP cells as previously reported [73,74]; similar responses were observed for E2 and this parallels the androgen-like activity of E2 in transactivation assays (Figs. 2–4). HPTE and ICI 182780 alone also increased AR levels but did not affect hormone-induced upregulation of AR protein. HPTE interactions with AR differ from the AR antagonist bicalutamide which downregulates AR and prevents DHT-induced upregulation of AR in LNCaP cells [73]. AhR agonists also blocked hormone-induced upregulation of AR protein and this paralleled the inhibitory AhR-AR crosstalk observed in transfection studies with pPB (Fig. 2). This suggests that modulation of AR protein by the AhR may contribute to inhibitory AhR-AR interactions; however, other factors, including promoter context, are important.

In summary, results of this study demonstrate that TCDD and the SAhRM 6-MCDF inhibit growth of LNCaP prostate cancer cells and inhibit hormone-induced upregulation of

AR protein. In contrast to AhR-dependent downregulation of ER α in breast cancer cells, AhR agonists alone did not affect AR levels in LNCaP cells and inhibitory AhR-AR crosstalk in transactivation experiments was promoter-dependent. These results suggest that ligand-dependent interactions between the AhR and AR signaling pathways are complex and current studies are investigating which key growth regulatory genes in LNCaP cells are targeted by the AhR. (Supported by Department of the Army DAMD17-02-1-0147).

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ARYL HYDROCARBON RECEPTOR AGONISTS INHIBIT HORMONE-INDUCED TRANSACTIVATION IN PROSTATE CANCER CELLS

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) are ligands for the aryl hydrocarbon receptor (AhR), where 6-MCDF is a weak AhR agonist for several TCDD-like toxic responses. This study investigated the interactive effects of AhR ligands and steroid hormones on receptor proteins, hormone-induced transcriptional activation, and proliferative responses in human prostate (LNCaP and 22RV1) cancer cells. Both LNCaP and 22RV1 cells express the androgen receptor (AR), and after treatment with 10 nM dihydrotestosterone (DHT), significant 2- to 4-fold upregulation of immunoreactive AR protein is observed within 3 h, and remains elevated for up to 24 h. DHT-induced upregulation of AR is inhibited in cells treated with TCDD or 6-MCDF. In prostate cancer cells transfected with a construct (PB-luc) containing an androgen-responsive probasin gene promoter insert, both TCDD and 6-MCDF significantly inhibited DHT-induced reporter activity, whereas this activity was not inhibited in cells transfected with a construct containing only the strongly androgen responsive region (ARR3TK-luc). Ten nM 17 β -estradiol (E2) also upregulated AR levels in LNCaP and 22RV1 prostate cancer cells and E2 significantly induced reporter gene (luciferase) activity in cells transfected with PB-luc or ARR3TK-luc. TCDD and 6-MCDF inhibited E2-induced activity only in cells transfected with PB-luc. E2-induced activity in cells transfected with PB-luc was inhibited by the estrogen receptor β (ER β) antagonist 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) and the pure antiestrogen ICI 182,780, whereas the latter compound did not inhibit DHT-mediated transactivation. These studies indicate that AhR-AR crosstalk is dependent on promoter context (i.e. PB vs. ARR3TK). Moreover, our results also suggest that E2-responsiveness of probasin may be ER β -dependent and the inhibitory effects of TCDD and 6-MCDF indicate that the AhR may inhibit ER β -induced genes in prostate cancer cells. (Supported by DAMD17-02-1-0147 and NIH ES09106)